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Nitric Oxide Production by Macrophages Stimulated with Coccidia Sporozoites, Lipopolysaccharide, or Interferon-γ, and Its Dynamic Changes in SC and TK Strains of Chickens Infected with Eimeria tenella

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SUMMARY. Nitric oxide (NO) is an important mediator of innate and acquired immunities. In the studies reported here, we quantified NO produced *in vitro* by chicken leukocytes and macrophages and *in vivo* during the course of experimental infection with *Eimeria*, the causative agent of avian coccidiosis, and identified macrophages as the primary source of inducible NO. *Eimeria tenella*–infected chickens produced higher levels of NO compared with noninfected controls. In *Eimeria*-infected animals, SC chickens produced greater amounts of NO compared with infected TK chickens, particularly in the intestinal cecum, the region of the intestine infected by *E. tenella.* Macrophages that were isolated from normal spleen were a major source of NO induced by interferon (IFN)-γ, lipopolysaccharide (LPS), and *E. tenella* sporozoites. Macrophage cell line MQ-NCSU produced high levels of NO in response to *Escherichia coli* or *Salmonella typhi* LPS, whereas the HD-11 macrophage cell line was more responsive to IFN-γ. These findings are discussed in the context of the genetic differences in SC and TK chickens that may contribute to their divergent disease phenotypes.

RESUMEN. Producción de ácido nítrico por macrófagos estimulados con esporozoitos de coccidia, lipopolisacáridos o interferón gama y sus cambios dinámicos en las líneas SC y TK de pollos infectados con Eimeria tenella.

El óxido nítrico es un mediador importante de la inmunidad innata y adquirida. Se cuantificó la producción de óxido nítrico producido *in vitro* por leucocitos y macrófagos de pollos e *in vivo* durante el curso de una infección experimental con *Eimeria,* el agente causal de la coccidiosis aviar, siendo identificados los macrófagos como la fuente primaria de óxido nítrico inducible. Se observaron niveles mayores de óxido nítrico en pollos infectados con *E. tenella* al ser comparados con los niveles observados en pollos control no infectados. En los pollos infectados con *Eimeria,* se observó una mayor producción de óxido nítrico en la línea SC al ser comparada con la línea TK, en especial en el ciego, región del intestino infectada por la *E. tenella.* Los macrófagos aislados a partir de bazos normales fueron la mayor fuente de óxido nítrico inducido por el interferón gama, lipopolisacáridos y esporozoitos de *E. tenella.* La línea celular de macrófagos MQ-NCSU produjo niveles altos de óxido nítrico en respuesta al lipopolisacárido del *Escherichia coli* o *Salmonella typhi,* mientras que la línea celular de macrófagos HD-11 mostró una mayor respuesta al interferón gama. Se discuten los hallazgos en el marco de diferencias genéticas entre las líneas de pollo SC y TK que puedan contribuir a la divergencia de sus fenotipos frente a las enfermedades.

Key words: nitric oxide, macrophage, coccidiosis, lipopolysaccharide, sporozoite, interferon-γ

Abbreviations: CMF-HBSS = calcium- and magnesium-free Hanks balanced salt solution; FCS = fetal calf serum; HEPES = *N*-[2-hydroxy-ethylpiperazin]-*N*′-[2-ethanesulfonic acid]; IFN-γ = interferon-gamma; IMDM = Iscove modified Dulbecco medium; LPS = lipopolysaccharide; MAb = monoclonal antibody; NO = nitric oxide; NOS = nitric oxide synthase; PBLM = peripheral blood leukocyte-derived macrophage; PBS = phosphate-buffered saline; PPI = post–primary inoculation; PSI = post–secondary infection

Nitric oxide (NO) is a multifunctional signaling molecule involved in a diverse array of biological processes, including vasorelaxation, antiaggregation of platelets, and neurotransmission (9,28,38). NO also is an important mediator of immunity and, as such, has been implicated in tumor cell toxicity (34) and antimicrobial activity against intracellular pathogens (14,29,30). NO is produced by oxidation of L-arginine by nitric oxide synthase (NOS). Three isoforms of NOS have been described: type 1 (neuronal NOS), type 2 (inducible NOS), and type 3 (endothelial NOS). Type 2 NOS is considered more important in immunity and inflammation (22). Macrophages and, to a lesser extent, leukocytes were reported as the major sources of inducible NOS (26,28). Cytokines such as interleukin (IL)-1β, IL-12, interferon (IFN)-γ, and tumor necrosis factor, superantigens; and bacterial lipopolysaccharide (LPS) are known to induce NO production (33).

Avian coccidiosis is an intestinal disease caused by several species of *Eimeria* protozoa. Because chicken peritoneal–derived macrophages and macrophage cell lines stimulated with LPS and/or cytokines produced NO (10,35,39) and since cytokines have been shown to play an important role in immunity to coccidiosis (17,19,39,40,41,42), several laboratories, including our own, have investigated the role of NO in the pathogenesis of this disease. For example, NO produced by chicken macrophages inhibited *E. tenella* replication *in vitro,* and this effect was reversed by N^G-monomethyl–L-arginine, a specific NOS inhibitor (5). In chickens that were experimentally infected with *E. tenella,* reduced fecal oocyst was observed in the presence of high levels of serum NO (1). However, our previous study, using two genetically divergent chicken strains that show different susceptibility to *E. tenella* (2), indicated that the production of serum NO during primary *E. tenella* infection may be only one of several immune responses and may not represent the main effector mechanism for innate resistance to *E. tenella* infection. Since *E. tenella* infection is mainly localized in the cecum, the measurement of local NO levels may be a better indicator of host innate immunity. The studies reported here were designed to expand upon these initial observations, specifically addressing the following questions: (1) Does local NO production correlate with the NO levels in the serum? (2) What subset(s) of chicken spleen cells produce NO *in vitro*? (3) Are the invasive stages of *E. tenella* (e.g., sporozoites and IFN-γ) capable of inducing NO *in vitro*? and (4) Do SC and TK chickens differ in their ability to produce NO *in vivo*, and, if so, is this difference related to their divergent degrees of pathogenesis to coccidiosis?

MATERIALS AND METHODS

Materials. All materials were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. Biologically active chicken recombinant IFN-γ was produced as described (20). Briefly, IFN-γ cDNA was subcloned into the pFastBac Hta vector (Life Technologies, Gaithersburg, MD), transformed into DH10Bac *E. coli* (Life Technologies), plasmid DNA gel purified, and transfected into *Spodoptera frugiperda* (Sf9) cells using CellFectin (Life Technologies), according to the manufacturer's instructions. Culture supernatant from transfected Sf9 cells served as the source of recombinant IFN-γ. Negative control supernatants were from nontransfected Sf9 cells.

Magnetic cell sorting. Spleens were removed aseptically and passed through a screen sieve into calcium- and magnesium-free Hanks balanced salt solution (CMF-HBSS); leukocytes were isolated by density gradient centrifugation (Histopaque 1077) at $400 \times g$ for 20 min at room temperature; and cells at the interface were washed three times with CMF-HBSS. Cell viability was consistently ≥95% by trypan blue exclusion. Spleen cells were suspended at 5×10^7 cells/ml in HBSS without phenol red containing 3% fetal calf serum (FCS) and 0.1% sodium azide, incubated on ice for 30 min with monoclonal antibodies (MAbs) specific for chicken CD4 (helper T cells), CD8 (cytotoxic T cells), or K1 (macrophage) antigens (13,18,21), washed three times, and incubated for 30 min on the ice with 100 μl of goat anti-mouse immunoglobulin G (IgG) antibody attached to latex microbeads (Miltenyl Biotech, Auburn, CA). Cells were washed three times and separated into the two major subpopulations of T lymphocytes, $CD4^+$ and $CD8^+$, and macrophages ($K1^+$) and nonmacrophages ($K1^-$) using an autoMACS cell sorter (Miltenyl Biotech). Purity of sorted subsets of cells was verified by staining. Postsorting cell viabilities were ≥95%.

Chicken macrophages. HD-11 and MQ-NCSU are two chicken macrophage cell lines transformed with avian myelocytomatosis virus MC 29 and Marek's disease virus, respectively (3,31). Both were seeded in 96-well flat-bottomed culture plates (Costar, Corning, NY) at 5×10^6 cells/ml in RPMI 1640 containing 10% FCS (HyClone, Logan, UT), 2.0 mM glutamine, 1.0 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 0.1 mM nonessential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 5.0 μg/ml 5-fluorocytosine, and 10 mM HEPES, pH7.3 (RPMI complete medium) at 40 C in 5% CO_2. To prepare peripheral blood leukocyte-derived macrophages (PBLMs), blood was collected by cardiac puncture from 3-mo-old specific-pathogen-free (SPA-

FAS) chickens in 10 U/ml heparin (Solopak, Franklin Park, IL), and PBLs were isolated by density gradient centrifugation, as described above. PBLs were seeded at 8×10^7 cells/ml in RPMI 1640 complete medium in six-well flat-bottomed plates, incubated for 24 hr at 40 C in 5% CO_2, and nonadherent cells in the supernatant were removed. Remaining adherent PBLMs displayed >99% staining with the macrophage-specific K1 MAb (13).

***In vitro* NO production.** Sorted $CD4^+$, $CD8^+$, $K1^+$, and $K1^-$ cells were resuspended at 1×10^6 cells/ml in RPMI complete medium and stimulated with 1×10^6/ml of *E. tenella* sporozoites, 2.5 μg/ml of *E. coli* O111:B4 LPS, recombinant chicken IFN-γ in supernatants of transfected Sf9 cells, or control nontransfected Sf9 cell supernatants. HD-11, MQ-NCSU, and PBLM cells were resuspended to 1×10^6 cells/ml in RPMI complete medium and stimulated with varying concentrations of LPS from *E. coli* O111:B4 or *S. typhi* or recombinant chicken IFN-γ in supernatants of transfected Sf9 cells. All cells were incubated at 41 C in 5% CO_2 for 48 hr and culture supernatants were collected and stored at −20 C until NO assay (see below).

***In vivo* NO production.** Specific-pathogen-free SC (B^2/B^2) and TK (B^{15}/B^{21}) chickens were obtained as fertilized eggs (HyLine International Production Center, Dallas Center, IA), hatched at the Animal and Natural Resources Institute (Beltsville, MD), raised in brooder batteries under pathogen-free conditions until 3 wk of age, and transferred to wire-mesh cages at 25 C–28 C with continuous light for experimentation. Animals were given broiler starter ration (Southern States Cooperative, Upper Marlboro, MD) and water *ad libitum*. *Eimeria tenella* sporozoites were prepared as described (25). Sporulated oocysts were collected from feces, cleaned with 5.25% sodium hypochlorite, ground with a rotary pestle to release sporocysts, and sporozoites were excysted in 0.125% (w/v) trypsin and 1% (w/v) taurodeoxycholic acid HBSS for 30 min at 41 C. Sporozoites were separated from cellular debris on DEAE-cellulose columns (Whatman, Clifton, NJ), as described (32). Three groups of 3-wk-old chickens (24/group) were orally inoculated with 1.0 ml of sterile deionized water (group 1) or 5×10^4 sporulated oocysts of *E. tenella* (groups 2 and 3). At days 1, 2, 3, 4, 6, 8, 10, and 12, three animals each in groups 1 and 2 were euthanatized and blood and tissue samples removed for NO assay (see below). At day 21 post–primary infection, group 3 chickens were reinoculated with 1×10^5 sporulated oocysts and samples removed according to the identical time schedule, as above.

Blood was collected by cardiac puncture and allowed to clot for 30 min at room temperature, and serum was separated by centrifugation at $1000 \times g$ for 10 min at 4 C and stored at −20 C until use. The intestinal duodenum and ceca were removed, cut longitudinally, and incubated separately in phosphate-buffered saline (PBS), pH 7.2, containing protease inhibitors (0.05 U/ml aprotinin, 5.0 mM ethylenediaminetetraacetic acid, and 2.0 mM phenylmethylsulfonyl fluoride) and 0.02% NaN_3 for 4 hr at 4 C with constant agitation on a rotating platform, as described (43). Intestinal washings were clarified by centrifugation at $3000 \times g$ for 20 min at 4 C, passed through 0.45-μm filters (Schleicher and Schuell, Keene, NH), and stored at −20 C until use. Splenic leukocytes were isolated as described above, washed three times in CMF-HBSS, and adjusted to 2×10^7 cells/ml in Iscove modified Dulbecco medium (Life Technologies) containing 10% FCS, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 5.0 μg/ml 5-fluorocytosine, and 10 mM HEPES, pH 7.3. Spleen leukocytes were stimulated with 2.5 μg/ml *E. coli* O111:B4 LPS or 1×10^6/ml *E. tenella* sporozoites for 48 hr at 41 C in 5% CO_2, and culture supernatants were clarified and stored as described above.

NO assay. Thawed samples were centrifuged at $1000 \times g$ for 30 min at 4 C, and 100 μl was mixed with an equal volume of freshly prepared Griess reagent containing 1% (w/v) sulfanilamine in 5% phosphoric acid and 0.1% (w/v) *N*-naphthylethylenediamine, incubated for 10 min at room temperature, and absorbency measured at 540 nm using a microtiter plate reader (BioRad, Hercules, CA). Nitrite concentrations were calculated from a standard curve using $NaNO_2$ (7). Experimental values were corrected by subtraction of background absorbency from culture medium alone or supernatants from nonstimulated cells.

Statistical analysis. Each treatment was analyzed in triplicate and data expressed as the mean ± standard deviation (SD). Assessment of the significance of differences between groups was performed by analysis of variance with Minitab software, version 10.1 (Minitab, State College, PA). Differences were considered significant at $P < 0.05$.

RESULTS

Macrophages are the major source of NO *in vitro*. Because leukocytes constitute a heterogeneous population and macrophages are the major source of inducible NO in mammals, we separated normal spleen leukocytes into $CD4^+$, $CD8^+$, $K1^+$, and $K1^-$ subsets, stimulated them *in vitro* with *E. tenella* sporozoites, *E. coli* LPS, or chicken IFN-γ, and measured NO in culture supernatants. As shown in Fig. 1, macrophages ($K1^+$) stimulated with recombinant chicken IFN-γ produced significantly greater amounts of NO compared with stimulation with control nontransfected Sf9 cell supernatant ($P < 0.01$). $CD4^+$ and $CD8^+$ cells stimulated with IFN-γ produced intermediate amounts of NO, but

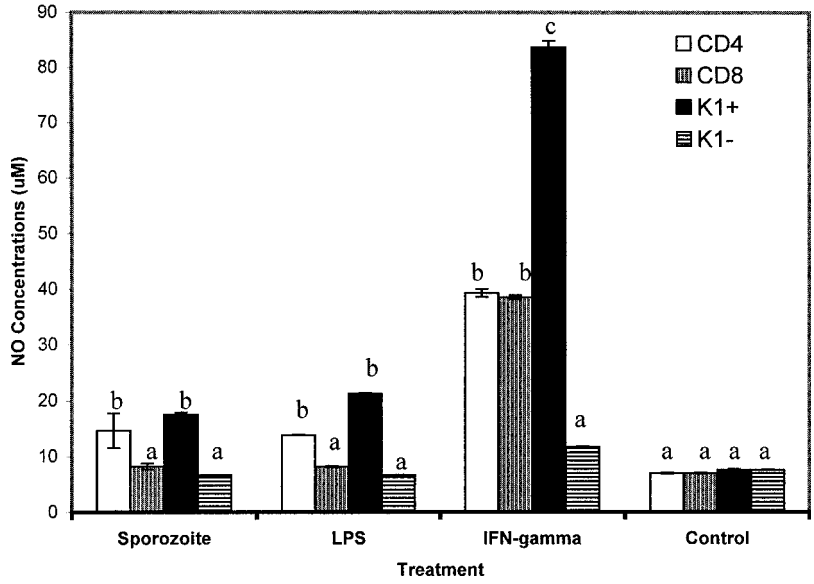

Fig. 1. NO produced by subsets of spleen lymphocytes. $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, macrophages ($K1^+$), and nonmacrophages ($K1^-$) were isolated from spleen cells by MAb-based fluorescence-activated cell sorting, stimulated with 1×10^6/ml of *E. tenella* sporozoites, 2.5 μg/ml of *E. coli* LPS, recombinant IFN-γ expressed in Sf9 cells or control nontransfected Sf9 cells, and NO concentrations in culture supernatants, determined as described in the Materials and Methods section. Data are expressed as mean ± SD of triplicate determinations. Bars with different letters show significantly increased NO compared with control at $P < 0.05$.

both values were significantly greater than that produced by stimulation with control supernatant ($P < 0.01$). $K1^+$ macrophages and $CD4^+$ cells stimulated with sporozoites or LPS also produced significantly more NO than control cells ($P < 0.05$). In summary, whereas normal macrophages consistently produced the greatest amounts of NO, $CD4^+$ and $CD8^+$ cells also could be induced to produce NO under certain conditions.

Evaluation of different stimulants for NO production *in vitro*. To confirm the above observations and to obtain a better understanding of the dose–response relationship between stimulation and NO production, two macrophage cell lines (HD-11 and MQ-NCSU) and normal macrophages isolated from PBLMs were stimulated with varying concentrations of LPS from *E. coli* or *S. typhi* or recombinant chicken IFN-γ and NO measured as above. As shown in Fig. 2A and B, MQ-NCSU cells stimulated with LPS from both sources produced significantly greater amounts of NO at all concentrations tested ($P < 0.05$). By contrast, recombinant chicken IFN-γ induced NO from HD-11 cells to a greater extent than from MQ-NCSU cells or PBLMs (Fig. 2C). Interestingly, while IFN-γ was an effective stimulant of NO from purified spleen macrophages (Fig. 1), it was relatively inefficient with PBLMs. HD-11, MQ-NCSU, and PBLMs stimulated with *E. tenella* sporozoites (1×10^6/ml) produced minimal levels of NO (1.78, 1.68, and 2.65 μM, respectively).

NO in serum, intestine, and spleen cell cultures from SC and TK chickens infected with *E. tenella*. Because NO is a mediator of cellular immunity (29,30,34), we were interested in quantifying its levels in SC and TK chickens infected with *Eimeria.* SC and TK chickens were given water (control) or were experimentally inoculated with 5×10^4 sporulated oocysts of *E. tenella*, and NO was measured in serum, duodenum, cecum, and spleen leukocytes stimulated by *E. coli* LPS or sporozoites at various times following primary infection. At 21 days post–primary infection, a third group of animals was reinfected with 1×10^5 oocysts and NO measured in an identical manner. Serum NO levels were identical in all noninfected animals and remained consistently less than 1.0 μM throughout the experimental protocol (unpubl. data). Following primary and secondary infections with *E. tenella,* increased levels of NO were seen in SC and TK chickens compared with noninfected animals. In general, serum NO con-

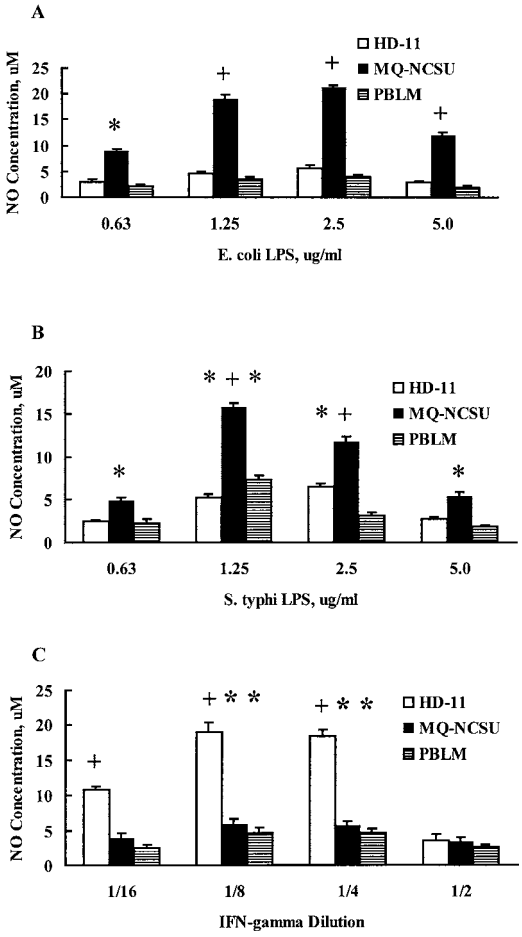

Fig. 2. NO produced by macrophages stimulated with LPS or IFN-γ. HD-11 and MQ-NCSU macrophage cell lines and PBLMs were stimulated with (A) *E. coli* O111:B4 LPS, (B) *S. typhi* LPS, or (C) recombinant IFN-γ expressed in Sf9 cells. Data are expressed as mean ± SD of triplicate determinations. Asterisks and crosses indicate significantly increased NO compared with control supernatants (*, $P < 0.05$; +, $P < 0.01$).

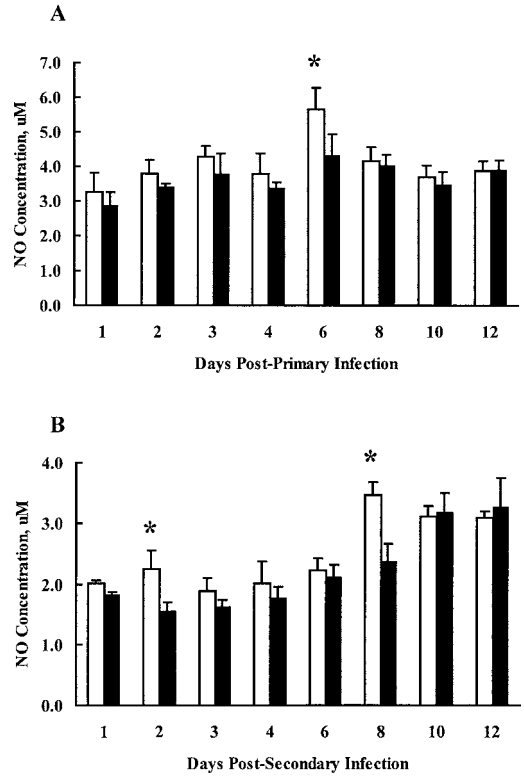

Fig. 3. NO in the serum of SC and TK chickens infected with *E. tenella*. (A) Chickens were orally inoculated with 5×10^4 sporulated oocysts at 3 wk of age and serum NO measured on the indicated days as described in the Materials and Methods section. (B) On day 21 post–primary infection, a second group of chickens was given 1×10^5 oocysts by the same route, and serum NO was measured on the indicated days. SC strain, open bars; TK strain, closed bars. Data are expressed as mean ± SD of triplicate determinations. Asterisks (*) indicate significantly increased NO ($P < 0.05$).

centrations were greater following primary infection compared with secondary infection (Fig. 3). However, with the exception of three isolated time points (day 6 post–primary infection and days 2 and 8 post–secondary infection), there were no significant differences in serum NO levels between infected SC and TK strains.

Because *Eimeria* are intestinal parasites known to display specificity in the region of the intestine they infect (19,40,41), we next examined NO concentrations in the duodenum and cecum of *E. tenella*–infected SC and TK chickens. As shown in Fig. 4, in the duodenum it appeared that there were two peaks of modest but significantly increased NO concentrations in SC, compared to TK chickens, at days 2 to 3 and 8 to 12 post–primary infection. Following secondary infection, duodenum NO levels displayed a single peak encompassing days 4 to 8. As shown in Fig. 5, this peak in NO production was more apparent in the cecum following primary and secondary experimental inoculations, in which case significantly greater NO levels were seen in SC chickens at days 8 to 12 and 4 to 10, respectively.

NO measurements in the spleen leukocytes stimulated *in vitro* with *E. coli* LPS (Fig. 6) or *E.*

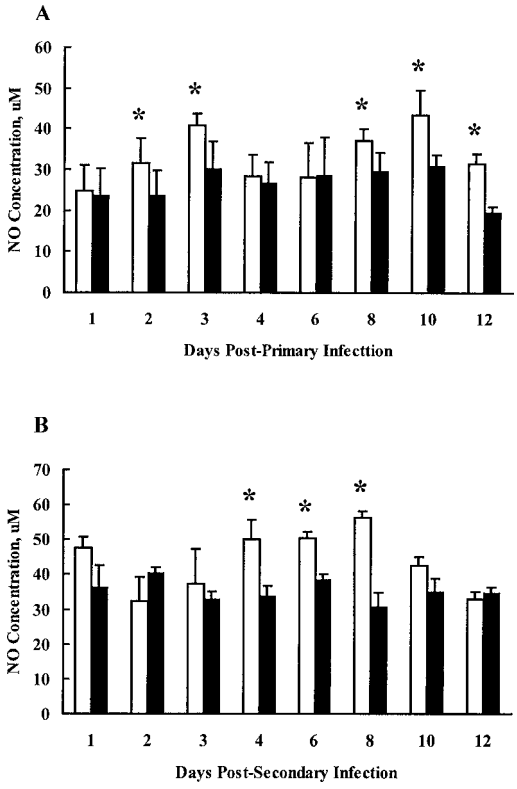

Fig. 4. NO in the duodenum of SC and TK chickens infected with *E. tenella*. Chickens were infected as described in Fig. 3, and NO in the duodenum was measured as described in the Materials and Methods section. (A) Primary infections; (B) secondary infections. SC strain, open bars; TK strain, closed bars. Data are expressed as mean ± SD of triplicate determinations. Asterisks (*) indicate significantly increased NO ($P < 0.05$).

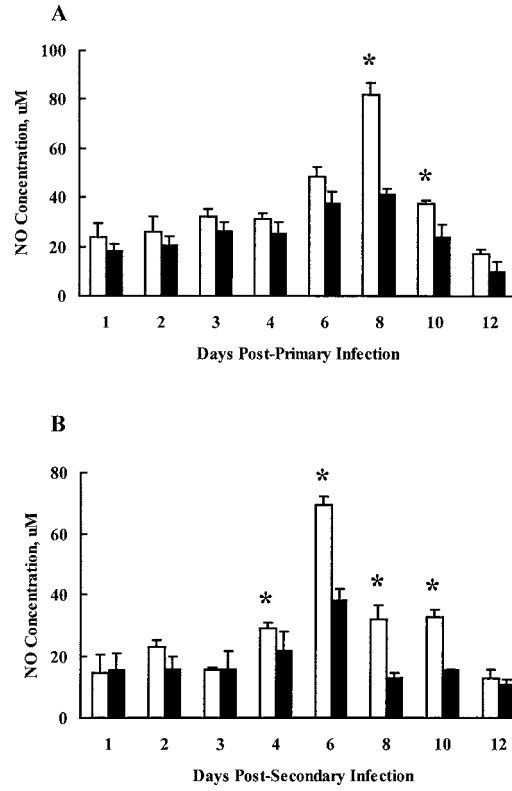

Fig. 5. NO in the cecum of SC and TK chickens infected with *E. tenella*. Chickens were infected as described in Fig. 3, and NO in the cecum was measured as described in the Materials and Methods section. (A) Primary infections; (B) secondary infections. SC strain, open bars; TK strain, closed bars. Data are expressed as mean ± SD of triplicate determinations. Asterisks (*) indicate significantly increased NO ($P < 0.05$).

tenella sporozoites (Fig. 7) showed that SC chickens produce higher levels of NO following primary infection. After secondary infection, LPS-stimulated spleen cells from SC chickens produced consistently enhanced levels of NO, whereas the sporozoite-induced spleen cells from TK chickens displayed significantly higher NO levels than SC chickens (e.g., days 2 and 12 post–secondary infection in sporozoite-stimulated cultures).

DISCUSSION

The results of our study are summarized as follows: (1) macrophages isolated from normal spleen were a major source of NO induced by IFN-γ, LPS, and *E. tenella* sporozoites; (2) the macrophage cell line MQ-NCSU produced high levels of NO in response to *E. coli* or *S. typhi* LPS, whereas HD-11 macrophage cells were more responsive to IFN-γ; and (3) *E. tenella*–infected SC strain chickens produced greater amounts of NO compared with infected TK chickens, particularly in the intestinal cecum, the region of the intestine infected by *E. tenella*.

As in mammalian species, chicken macrophages play important roles in innate and adaptive immunities (14,15,23,35), notably in response to intracellular parasites such as *Leishmania* (16,29) and *Eimeria* (1,5). One of the more important manifestations of the antimicrobial activities of macrophages is production of NO (22,33,36), although this property is not exclusive to macro-

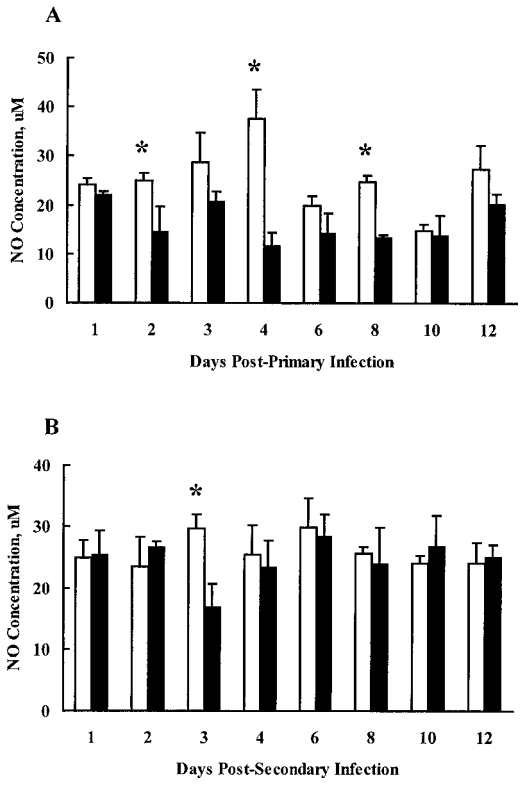

Fig. 6. NO in supernatants of spleen cells stimulated with *E. coli* LPS. Chickens were infected as described in Fig. 3, and NO in spleen lymphocyte cultures was stimulated with 2.5 μg/ml of *E. coli* LPS measured as described in the Materials and Methods section. (A) Primary infections; (B) secondary infections. SC strain, open bars; TK strain, closed bars. Data are expressed as mean ± SD of triplicate determinations. Asterisks (*) indicate significantly increased NO ($P < 0.05$).

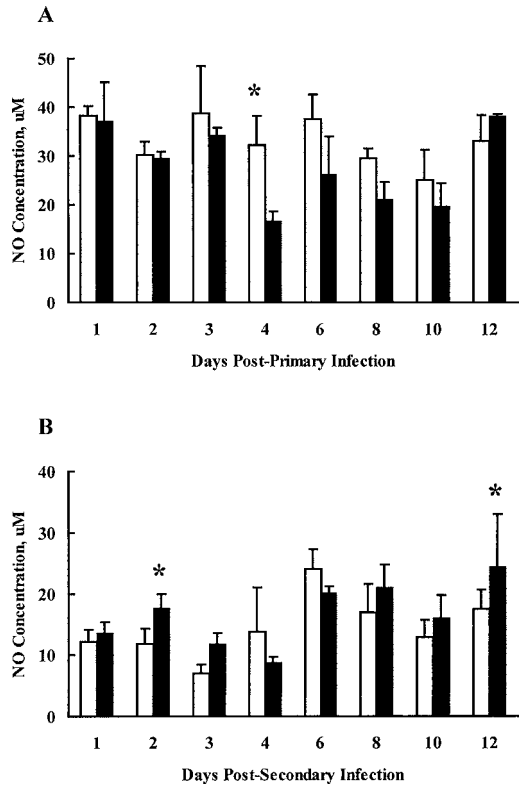

Fig. 7. NO in the culture supernatants of spleen cells stimulated with *E. tenella* sporozoites. Chickens were infected as described in Fig. 3, and NO in spleen lymphocyte cultures stimulated with 10^6/ml of *E. tenella* sporozoites was measured as described in the Materials and Methods section. (A) Primary infections; (B) secondary infections. SC strain, open bars; TK strain, closed bars. Data are expressed as mean ± SD of triplicate determinations. Asterisks (*) indicate significantly increased NO at $P < 0.05$.

phages, since other cell types, including leukocytes (26) and epithelial and mesenchymal cells (38), are also capable of induced NO synthesis. We previously observed that chicken spleen leukocytes stimulated *in vitro* with *E. tenella* sporozoites produced NO (1,2). The results of the studies reported here corroborate the role of chicken macrophages in NO production induced by *Eimeria* sporozoites, bacterial LPS, and chicken IFN-γ. The latter, shown in mammalian systems to be a powerful NO stimulant (6), also induced NO from $CD4^+$ and $CD8^+$ chicken spleen leukocytes.

Although the ability of cytokines or bacterial components to induce NO by macrophages is well established, some debate remains concerning the necessity for single or combination signal stimulation. Some studies showed that macrophages did not generate NO following stimulation by the first signal alone (i.e., the stimulant used to elicit macrophages from the peritoneum) but instead became primed, such that NO was produced following a second signal. For example, Stuehr and Marletta (33) reported that mouse peritoneal–derived macrophages activated *in vivo* by intracellular pathogens such as *Mycobacterium bovis* or *in vitro* by lymphokines produced NO only after an additional stimulation by LPS. On the contrary, other studies demonstrated that LPS alone was sufficient to stimulate the generation of NO by peritoneal-derived macrophages and macrophage cell lines (11,12,35,36,37). In our study, we found that macrophage cell lines and PBLMs produced

NO in response to stimulation *in vitro* by sporozoites, LPS, or IFN-γ.

Chicken peritoneal–derived macrophages and cell lines (HD-11 and MQ-NCSU) have been demonstrated to produce NO in time- and dose-dependent manners in response to LPS and/or lymphokines (10,11,35,36). Using a high dose of LPS (5.0 μg/ml), highest levels were produced following 20 to 24 hr of culture. At a lower concentration (5.0 ng/ml), NO production was delayed for an additional 24 hr. Karaca *et al.* (12) showed that HD-11 macrophages stimulated with 0.25–2.0 μg/ml of LPS produced maximum concentrations of NO when cultured for 48 hr. Our dose–response studies also revealed highest NO levels induced by 1.25–2.5 μg/ml of LPS, particularly when using MQ-NCSU cells cultured for 48 hr. Concentrations of LPS below or above these values tended to stimulate lower levels of NO. The delayed effect of LPS on NO production may be related to the fact that relatively lengthy incubation times are required for LPS to induce phosphorylation and activation of Stat1α, a transcription factor for the type 2 inducible NOS gene (6).

The antimicrobial properties of NO, well appreciated in mammalian species, prompted us to examine its role in experimental avian coccidiosis. The SC/TK inbred chicken model system provides a unique opportunity to assess *in vitro* parameters of protective immunity to this disease (18,19,43). SC and TK inbred chicken strains display differential protective immunity to *E. acervulina* (4,24) and *E. tenella* (42), SC being more resistant and TK more susceptible to disease. In this regard, several studies have shown that enhanced disease resistance by SC chickens is associated with more active cell-mediated immune responses against coccidia parasites. We previously reported that cell-mediated immunity against *Eimeria* parasites was enhanced in SC compared with TK chickens with respect to activation of lymphocyte subsets (18,42) and cytokine production (4,8,40,41,42,43). Notably, significantly higher expression of IFN-γ (4,42), IL-2 (27), and increased percentages of $CD4^+$ and $TCR1^+$ T lymphocytes both in the intestine and spleen (18,42) were seen in *Eimeria*-infected SC chickens compared with stain TK. Allen and Lillehoj (2) reported that SC strain chickens produced higher level of plasma NO during primary and secondary anti-coccidia immune responses compared with strain TK. Our findings confirm and extend these studies, further demonstrating the local production of NO following *E. tenella* infection. Since chicken IFN-γ induced NO *in vitro,* it is not surprising that SC chickens with higher levels of IFN-γ during coccidiosis (42,43) also produced more NO *in vivo.* Although chicken macrophages have been shown to play an important role in response to *Eimeria* parasite infections (15,18), no quantitative differences in SC and TK strain macrophages have been reported. Our results, however, imply that qualitative differences exist between these two strains, particularly in terms of the ability of spleen macrophages to produce NO. Experiments are currently underway in our laboratory to further explore NO production by SC and TK strain macrophages, with the long-term goal of developing immunotherapeutic regimens to control avian coccidiosis in commercial poultry flocks.

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